OXYGEN CONSUMPTION AND ITS INHIBITION IN THE DEVELOPMENT OF FUNDULUS AND VARIOUS PELAGIC FISH EGGS

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Introduction

Loeb (1895) has shown that during the first few days after fertilization the egg of *Fundulus heteroclitus* is capable of extensive development under anaerobic conditions in contrast to the immediate suppression produced in the developing *Ctenolabrus* egg. Anaerobic development in amphibian eggs has also been observed by earlier workers and has recently been confirmed by Brachet (1934) in *Rana fusca* and *Discoglossus* eggs with the use of HCN as a respiratory inhibitor. Cyanide, like other hemochromogen-binding reagents such as NaN_3 and CO, acts as a respiratory inhibitor by combining in a specific, reversible manner with respiratory catalysts like cytochrome-oxidase (Keilin, 1933). The activity of these three reagents has already been used to advantage on the *Fundulus* egg for demonstrating the presence of cytochrome-oxidase in the chain of reactions which control the frequency of the embryonic heart-beat (Fisher and Cameron, 1938; Armstrong and Fisher, 1939; Fisher and Ohnell, 1938). If the respiratory systems of both *Fundulus* and *Ctenolabrus* eggs are also sensitive to cyanide and azide, the effect of these substances on the development of these eggs ought to be analogous to the effect of anaerobiosis. Therefore it would be of interest to apply a similar sort of analysis as that of Fisher and co-workers to the development of the embryos of these fishes and to compare the results obtained with those of Loeb.

Various studies have been made of the respiration of the *Fundulus* egg during early development. Boyd (1928) has reported a striking but temporary increase in respiration shortly after fertilization. Hyman (1921) has found that there are fluctuations in respiratory rate during *Fundulus* development, and the studies of Trifonova (1937) have shown similar changes in the perch. However, such fluctuations are not found in amphibian eggs where increases in respiratory rate have been shown to occur continuously by Brachet (1934), Atlas (1938), and Steffanelli (1938). Both Hyman and Trifonova have attempted to

relate these changes in the fish embryos to specific morphological processes.

A careful study of the respiration of the *Fundulus* egg throughout development to hatching has been made by Amberson and Armstrong (1933); but since their method of measurement did not allow for the detection of possible fluctuations in respiratory rates within daily intervals, it would seem of value to re-study the same problem during early development by another method.

This paper deals with the two problems, (1) the respiration of the *Fundulus* egg during the first two and one-half days of development and its sensitivity to NaCN, and (2) the comparison of the development of the *Fundulus* egg with that of several pelagic, fish eggs in various concentrations of NaN_3 and NaCN.

Materials and Methods

Fertilized eggs of *Fundulus heteroclitus* and of the cunner, *Tautogolabrus adspersus* (*Ctenolabrus*), were obtained by stripping sexually mature females and males. The eggs of mackerel, *Scomber scombrus,* and scup, *Stenotomus chrysops,* were provided by the U. S. Bureau of Fisheries within a few hours after stripping. All eggs were allowed to develop at laboratory temperatures (20–25° C.).

For the measurements of respiration Fenn volumetric micro-respirometers were used. The apparatus had capillary volumes of about 0.8 cu. mm. and 2.0 cu. mm. per cm. of length. Apparatus constants calculated according to the Fenn (1928) equation were respectively about 0.9 and 2.4 cu. mm. per cm. of capillary length. These sensitivities were obtained by modifying the sizes of the respirometer vessels so that the ratio of control to experimental gas spaces was about 4/1 (27 ml. to 7 ml.). The respirometers were shaken in a water-bath maintained at 22° C. at the rate of 110 times per minute through an arc whose chord was 7.5 cm. The carbon dioxide produced was absorbed by molar KOH in the side-arm of the experimental vessel. The volume of the eggs and sea water was always brought to 1 ml.

The respiration measurements were made on fertilized eggs immediately after they had been washed in sea water to remove excess sperm. The eggs were counted at the end of each experiment and the data were rejected if more than 10 per cent of the eggs were abnormal or unfertilized. In experiments on later embryological stages, however, only normal-appearing eggs were used. Between 50 to 100 eggs were used in each experimental vessel. Eggs once used for a cyanide experiment were discarded. The determinations of the cyanide sensitivity of the

respiration were all carried out in M/1,000 NaCN in sea water. The NaCN solutions were adjusted to the pH of sea water with HCl. In these determinations the KOH of the side-arm was replaced by Krebs' (1935) KCN–KOH mixture. No correction was made for the use of this mixture with sea water and it is possible that the total concentration of cyanide in the solution surrounding the eggs was greater than M/1,000. In order to determine the cyanide-insensitive respiration accurately it was necessary to use about twice as many eggs as in the experiments with unpoisoned controls. All respiratory rates are given in cu. mm. O_2/egg/hour.

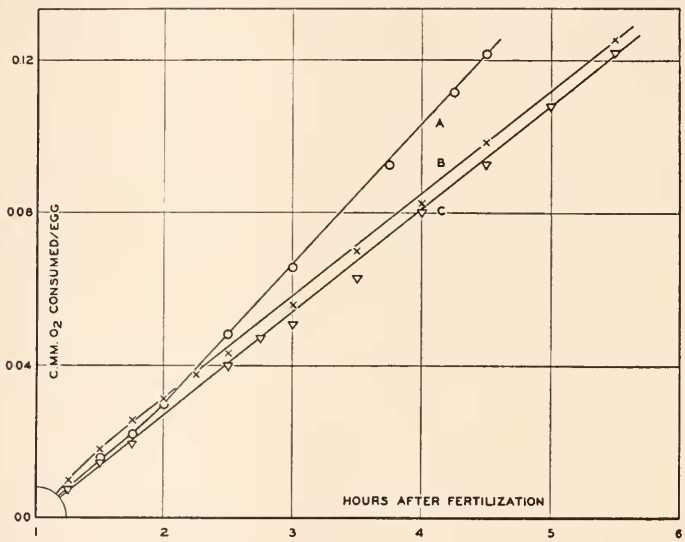

FIG. 1. Oxygen consumption of fertilized *Fundulus* eggs. For curves *A*, *B*, and *C*, respectively 75, 58, and 94 eggs were used.

To determine the effects of NaCN and NaN_3 on the development of *Fundulus*, 50 to 100 fertilized eggs were placed in 150-ml. Erlenmeyer flasks with 25 ml. of freshly prepared solution. The eggs were well covered; but the solution was shallow enough so that an adequate supply of oxygen was available. The solutions were changed three times daily. In the experiments with pelagic eggs several milliliters of a concentrated suspension of fertilized eggs were added to each flask with 50 ml. of sea water solution. Since the eggs float on the surface, there is no problem of oxygen diffusion through the solution. With these eggs the rapidity of development obviated the necessity of prolonged observation and therefore no change of solution was made. The

NaCN solutions were made up in sea water and brought back to the pH of sea water with HCl. As the pH of the NaN_3-sea water solutions is lowered, the concentration of HN_3 increases resulting in higher concentrations of azide within the egg. Thus the inhibitory effect of NaN_3 solutions is increased by lowering the pH (Keilin, 1936; Armstrong and Fisher, 1939). For this reason the NaN_3 solutions were made up with sea water which had been acidified with HCl and equilibrated by passing a strong stream of air through it for several hours. In a few experiments sea water buffered with M/100 phosphate buffer was used; but this method is complicated by the deposition of insoluble phosphate salts in experiments of duration longer than about 12 hours. The pH values were determined with the glass electrode.

In recording the stages of development of the *Fundulus* eggs in these respiratory-inhibitor experiments fifty or more eggs from each solution were examined with the aid of a binocular dissecting microscope. The stages were designated according to Oppenheimer (1937), and then the eggs were returned to the original solutions. In the case of the pelagic eggs a sample portion of those still floating was removed from the solutions and the stage found representative of the majority of at least 30 living eggs was recorded. This presented no difficulty since the pelagic eggs no longer float after dying.

Experimental Results

The Respiration of the Fundulus Egg

The respiratory rate within an hour after fertilization appears to have a more or less constant value as shown in Fig. 1. In these experiments the rates of oxygen consumption were far above the limit of sensitivity of the apparatus. They involved indicator drop movements of 1.5 to 3 cm. per hour. There is no evidence for a significant rise in respiratory rate to a maximum at about 90 minutes after fertilization with a subsequent decline as expected from Boyd's results (Table I).

In Fig. 2 are plotted a number of individual experiments carried out on eggs at various times during the first two days of development. The complete graph of all the determinations of respiratory rates of unpoisoned eggs is shown in the upper curve of Fig. 3. The individual points in the upper curve of Fig. 3 are the rates per egg per hour calculated for periods of 1–2 hours duration. No more than two points are taken from a single experiment and these are separated by an interval of at least four hours.

The curve which is drawn through these points has been fitted to the averages of all the rates within successive three-hour periods. No

emphasis is to be placed on the exact nature of the curve. The character of the break which is shown cannot be accurately determined from the data at hand. However, it is clear that after about 15 hours of development a change occurs in the rate of increase of oxygen consumption.

Figure 2 shows that the rate of oxygen uptake increases with advance in development of the fertilized egg. This is also brought out in Fig. 3 where the actual rates of respiration per egg can be seen to rise with the increase in time after fertilization. Further, in the individual experiments of Fig. 2 and in the composite of results in Fig. 3 the rise

TABLE I

Rate of oxygen consumption of fertilized *Fundulus* eggs. Calculations are for successive half-hour periods after fertilization. Rates are given as cu. mm. O_2 consumed/egg/hour.

No of Eggs	Percentage Abnormal	Hours after Fertilization								
		1–1½	1½–2	2–2½	2½–3	3–3½	3½–4	4–4½	4½–5	5–5½
78	1	—	—	.029	.035	.038	.037	.044	.054	—
108	9	—	—	—	—	—	.029	.022	.029	.026
94	5	.029	.026	.026	.022	.032	.036	.027	.032	.026
75	4	.032	.028	.037	.035	—	.036	.039	—	—
66	3	—	.033	.035	.030	—	.031	.037	—	—
58	9	.036	.027	.024	.025	.028	.025	.032	.027	.027
51	0	.036	.027	.023	.023	.036	.026	.025	.040	.040
Averages		.033	.028	.029	.028	.034	.031	.032	.036	.030

in the rate of oxygen consumption in the period between six and fifteen hours after fertilization is more rapid than it is before six or after fifteen hours. Moreover, during the six to fifteen-hour period the embryo is advancing from the very early high blastula to the flat blastula stage.[1] The morphological changes which do occur during this segmentation period involve a large increase in cell number, and it is probable that this is directly associated with the rapid increase in respiratory rate. There is no evidence, from the data presented in Figs. 2 and 3, of any major fluctuations in respiratory rate which might possibly be

[1] The description of the normal stages of *Fundulus* is taken from Oppenheimer (1937).

correlated with definite morphological events such as the beginning of gastrulation. On the contrary, there is a continuous, if not constant, increase in respiratory rate with advance in embryological differentiation.

Considering the differences in the two methods of measurement there is fairly good agreement between the results presented in Fig. 3 for the first two days after fertilization and those of Amberson and Armstrong. They found rates between 0.7 to 0.9 and 1.9 to 2.2 ml./day/1,000 eggs for the first and second day, respectively. By appropriate change of

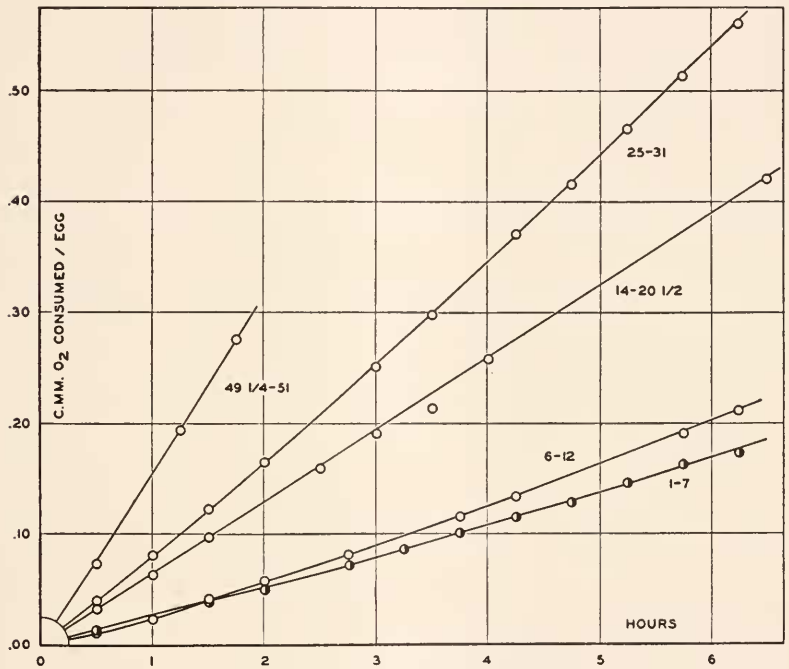

FIG. 2. Oxygen consumption of developing *Fundulus* eggs. Figures adjacent to curve indicate time in hours after fertilization.

units and by summation of graphically determined values for successive three-hour periods the present data give values of about 1.2 and 2.6 ml./day/1,000 eggs.

The Cyanide Sensitivity of Fundulus Respiration

During the first six hours after fertilization the respiration of *Fundulus* eggs in M/1,000 NaCN solutions was found to be 32 per cent of the average normal respiration. In order to ascertain the cyanide-stable respiration of later stages, eggs were allowed to develop in normal sea water until the desired stages were reached. They were then placed

in the cyanide medium and their oxygen consumption determined for a period of at least two hours. The maximum respiratory inhibition was always attained within less than an hour after transfer to the M/1,000 NaCN. The results of these experiments are shown in the lower curve of Fig. 3. If the two curves of Fig. 3 are compared, it becomes evident that the cyanide-resistant respiration increases slightly in rate within the first day and then remains constant throughout the next day and a half. Actually, in two determinations at 98 hours after fertilization the

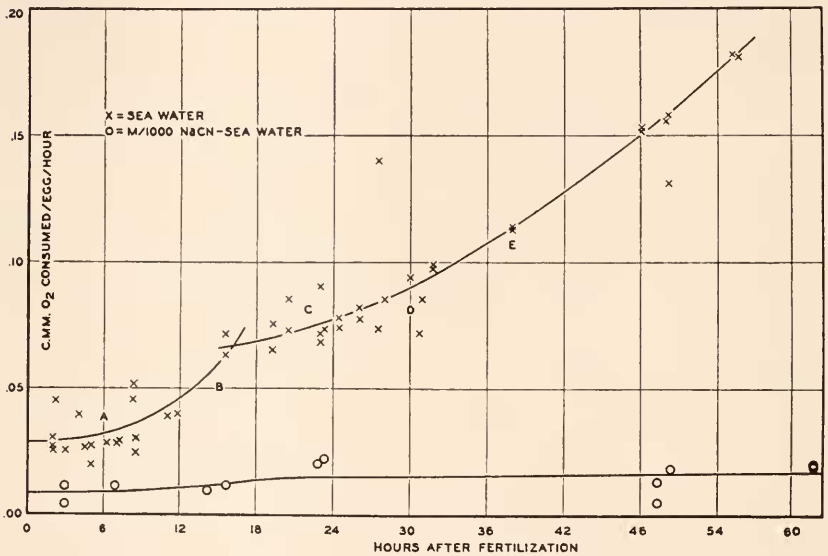

Fig. 3. Rate of oxygen consumption during the first two and one-half days of development of *Fundulus* in untreated sea water and in M/1,000 NaCN. *A*, early high blastula; *B*, flat blastula; *C*, early gastrula; *D*, late gastrula; *E*, appearance of optic vesicles. For the determination of the cyanide-sensitivity of different stages of development eggs were raised in normal sea water to the desired stage, transferred to cyanide sea water in the respirometers, and rates determined over two-hour periods.

cyanide-stable respiration had still remained unchanged, viz., 0.020 and 0.022 cu. mm./egg/hour, as compared to .019 cu. mm./egg/hour at about 62 hours. The increased respiration during the first four days of development may thus be seen to be almost exclusively the result of a cyanide-sensitive respiratory system. That this is the cytochrome-oxidase system is expected from the work of Fisher and co-workers.

The Effect of Respiratory Inhibitors on the Development of Fundulus

So far it has been shown that the inhibition of respiration of the *Fundulus* egg in M/1,000 NaCN is maximal within an hour after trans-

fer from normal sea water. It is of interest now to investigate the effect of NaCN-sea water solutions on the development of the egg. In contrast to the immediate effect produced by NaCN on their respiration, eggs placed in solutions of M/1,000 NaCN approximately one-half hour after fertilization and prior to the completion of the blastodermic cap, developed to late high and flat blastula stages before coming to a complete halt. The rate of development of such eggs is not much slower than the controls. In one experiment at 20 hours after fertilization, eggs in M/1,000 NaCN were found to be in the late high or flat blastula stages (9 and 10 of Oppenheimer). The sea water controls were mostly in the flat blastula stage with only about one-fifth already expanding blastulae (Stage 11). This is a difference of less than six hours of normal development. It is very probable that the eggs in M/1,000 NaCN had reached a state of complete developmental inhibition several hours before the time of observation which might account for most of the difference. Subsequent observations on the same inhibited eggs at about 33 and 48 hours after fertilization revealed no further development. Thus it is clear that eggs in M/1,000 NaCN continue to develop from shortly after fertilization to the late blastula stages before they are completely inhibited. Further, the rate of this development in M/1,000 cyanide is not much slower than that of control eggs in normal sea water.

The effects of other concentrations of NaCN on the development of eggs soon after fertilization are shown in the results of a similar experiment (Table II). At 22 hours the eggs in the most concentrated NaCN solution were in stages attained by untreated eggs about eight hours earlier. Twenty-four hours later the eggs in M/2,000 NaCN had developed no further than normal eggs do in about six hours. From then on the eggs in M/2,000 NaCN exhibited little, if any, significant development. Altogether, these eggs have undergone a total development equivalent to that found in untreated eggs in about 20 hours. By two days it is also seen that the eggs in M/2,000 NaCN have reached the maximum development possible. In M/8,000 and M/16,000 NaCN the eggs continue to develop beyond the two-day period but more slowly than the controls.

The experiments described above have shown that eggs, transferred to the higher concentrations of NaCN, M/1,000 or M/2,000, within a few hours after fertilization, are able to develop to the beginning of gastrulation. This might possibly indicate that at this stage there exists an increased sensitivity to the presence of high concentrations of NaCN. To test this possibility eggs were allowed to develop in normal sea water until the flat and expanding blastula stages (10 and 11) and then transferred to NaCN solutions. Such eggs in M/2,000 NaCN became middle

gastrulae (Stage 13) before development was suppressed. This is equivalent to the amount of development occurring in normal eggs in about 10 hours. Correspondingly, 71 hours after fertilization eggs in M/4,000 NaCN were still in the stage of blastopore-closure (Stage 15); in M/8,000 NaCN embryos had developed hollow optic vesicles (Stage 17); but in the M/16,000 solution development was beyond the recorded

Table II

Development of *Fundulus* eggs in NaCN. Experiment started about three hours after fertilization when eggs were still in two-cell stage. Observations were made after 22, 48, and 71 hours.

Hours after Fertilization	Concentration of NaCN	Total No. of Eggs Observed	Percentage Abnormal or Dead	Oppenheimer Stages of Fundulus Egg								
				9	10	11	12	13	14	15	16	17
22	M/2000	67	13	21	37							
	M/4000	50	24	2	36							
	M/8000	62	39		1	37						
	M/16,000	74	39		1	43	1					
	Control *	65	32		1	21	22					
48	M/2000	65	37			38	3					
	M/4000	61	33				8	36				
	M/8000	62	45						2	26	6	
	M/16,000	68	40								14	27
71	M/2000	63	51			20	11					
	M/4000	65	35				41	1				
	M/8,000	76	42						■	2	19	23

* No further observations of control eggs are included in table since at the later times the control eggs had developed beyond the stages recorded in these experiments.

stages. It is clear from such data that eggs which are already beginning to gastrulate are still capable of considerable development under conditions of reduced respiration. Furthermore, these eggs develop beyond stages at which suppression occurred when cyanide was used from the beginning of development. This indicates that there are no specific morphological stages at this period of development where sensitivity to

cyanide poisoning (i.e. to reduced cellular oxidations) is particularly evident. However, the older eggs are able to develop for a shorter duration of time, measured from the beginning of cyanide treatment, than are the younger eggs.

It is important to consider the possible relationship between suppression of development and death of eggs in the NaCN solutions. In Table II it will be noted that the percentage of eggs which are recorded as abnormal or dead increases during the course of the experiment. This might be interpreted as indicating that the suppression of development manifests itself in the death of eggs at a stage of development of increased sensitivity to the poison.

There are, however, several reasons for not accepting such an interpretation. In the first place, an inspection of Table II reveals that at 22 hours there is no obvious connection between the concentration of NaCN and the number of dead eggs. In fact, their number in the control is greater than in M/2,000 or M/4,000 NaCN. Further, eggs recorded as existing in arrested states of development are actually alive, as was tested by transferring them into normal sea water which removes the cyanide inhibition and allows development to continue. After several days in normal sea water, it was found that the percentage of developing eggs was the same as the percentage of those which had been considered in arrested states of development during NaCN treatment. Of all the eggs previously in M/2,000, M/4,000 and M/8,000 NaCN (Table II) respectively 46, 51, and 71 per cent were developing.[2]

There are additional objections to the interpretation that suppression of development is the result of the death of the eggs in stages of increased sensitivity to cyanide. One of these is the fact that the percentages of abnormal or dead eggs did not necessarily increase during the period of complete inhibition in every experiment (see Table III). Moreover, it has already been pointed out that during the period of development studied in this paper there is no evidence for an increased cyanide-sensitivity at any particular developmental stage.

The results with solutions of NaN_3 are essentially similar to those with NaCN (Table III). Results with eggs transferred to NaN_3 from normal sea water in the middle gastrula period (Stage 13) were also similar to those obtained with NaCN.

[2] Many of the eggs which develop further after the extended developmental arrests in the poison solutions show numerous abnormalities. This may be expected from any inhibitory treatment of the early embryonic stages of the *Fundulus* egg (Stockard, 1921). No analysis of these abnormalities was made since the primary interest here was whether the previously inhibited eggs were still capable of development.

The Effect of Respiratory Inhibitors on the Development of Pelagic Eggs

When experiments similar to those described above were carried out with some pelagic fish eggs, entirely different results were obtained. If, for instance, cunner eggs in the two-cell stage were transferred to M/1,000 NaCN solution, they did not develop beyond the formation of the second cleavage plane. This state was reached by them and by the

TABLE III

Development of *Fundulus* eggs in NaN_3. At time of making up fresh solutions pH = 6.2–6.8. Experiment started about three hours after fertilization when eggs were in four- and eight-cell stages.

Hours after Fertilization	Concentration of NaN_3	Total No. of Eggs Observed	Percentage Abnormal or Dead	Oppenheimer Stages for Fundulus							
				9	10	11	12	13	14	15	16
47	M/500	50	40	11	19						
	M/1000	47	21		6	27	4				
	M/2000	41	20			8	25				
	M/5000	39	13				4	30			
	M/10,000	43	16						2	34	
70	M/500	51	41	7	20	3					
	M/1000	53	23		9	32					
	M/2000	42	26			8	23				
	M/5000	44	23					34			
	M/10,000	47	11						1	26	15

control eggs within 20 minutes. Similarly (Table IV) in M/10,000 NaCN, eggs never developed beyond the stage reached by the controls in one-half hour. Moreover, late blastulae or early gastrulae, when treated with M/10,000 NaCN, never advanced beyond these stages.

Further examination of Table IV reveals that in M/20,000 and M/40,000 NaCN the cunner eggs were able to proceed to later stages before complete inhibition resulted. However, the rate of their development to these stages was definitely less than that of the control eggs. A still different situation existed in M/80,000 and M/100,000 NaCN solutions where the eggs showed a decrease in rate of development only at a

relatively later time and never were inhibited completely. As early as 4½ hours and 7 hours the eggs in M/80,000 appeared qualitatively as somewhat retarded late blastulae as compared with eggs in M/100,000 NaCN, or in the control flask. Such results are not to be interpreted as arising from the effect of decreased rates of penetration of NaCN from the less concentrated solutions. It has been shown above that eggs in the more dilute concentrations demonstrate the presence of cyanide internally by retardation in development for a considerable length of time before complete developmental arrest takes place. Furthermore, an in-

TABLE IV

Development of cunner eggs in NaCN. Experiment started one-half hour after fertilization when eggs were still in stage of formation of blastodermic cap.

Concentration of NaCN	Hours after Addition of NaCN						
	½	1	1¾	4½	7	20	27
M/10,000	2 cells	2 cells	2 cells	disintegration			
M/20,000			4 cells	32–64 cells	disintegration		
M/40,000			8 cells	early high blastula	early high blastula	disintegration; early high blastula	
M/80,000			16 cells	late high blastula	late high blastula	2/3 gastrula	embryo 1/2 around yolk
M/100,000			16 cells	late high blastula	late high blastula	2/3 gastrula; closure of blastopore	embryo 2/3 around yolk
Control	2 cells	4 cells	16 cells	late high blastula	late high blastula	10 somites; embryo 1/2 around yolk	embryo 5/6 around yolk

crease in sensitivity to NaCN during development will not account for the inhibitions. Experiments show that eggs transferred from normal sea water to NaCN after having reached the late blastula stage are still capable of undergoing gastrulation for several hours in such concentrations as M/40,000.

Several hours after complete inhibition of development had occurred in these pelagic eggs disintegration became evident. The cell walls of early cleavage stages appeared to dissolve resulting in a completely undivided blastodermic cap which eventually became opaque. At the same

time shrinkage of the yolk occurred and the eggs no longer floated at the surface of the solution. If later stages were attained before complete inhibition, an increasing disorganization and opacity of the embryonic tissues with yolk shrinkage and loss of buoyancy occurred. All these observations are in complete agreement with those that Loeb obtained on *Ctenolabrus* (cunner) under anaerobic conditions.

The results of experiments with NaN_3 on cunner eggs were similar to those obtained with NaCN. It is interesting to note that the concentrations of NaN_3 required to inhibit the development of the cunner eggs were about ten times higher than those treated with NaCN (Table IV). At present there is no available explanation for this observation.

In the earlier part of the summer it was possible to carry out a few experiments on mackerel eggs. The concentrations of NaCN used, ranging from M/200 to M/10,000, inhibited development equally rapidly. Eggs poisoned during the second cleavage proceeded to fourth cleavage while those in the third proceeded to the fifth cleavage. In both cases this was equivalent to the development occurring in the control eggs in one hour. Moreover, the experimental were somewhat slower than the control eggs in reaching the limit of their development. Eggs of later stages were equally sensitive.

When eggs in the second cleavage stage were transferred to unneutralized sea water, M/100 with respect to NaN_3, cleavage proceeded only to 32 cells before being completely inhibited. M/1,000 NaN_3 under similar conditions stopped development in the early gastrulation stages. By using sea water, buffered to lower pH (6.2–6.3) with phosphate salts, it was possible to inhibit the eggs of the 2-cell stage within one cleavage with M/1,000 NaN_3. Even lower concentrations produced complete inhibition.

In one experiment with scup eggs at two different stages NaN_3 was used in phosphate-buffered sea water. Beginning with the one-cell stage eggs in M/1,000 proceeded as far as 4 to 16 cells while in M/10,000 inhibition was not complete until the late high blastula stage. Scup eggs transferred to M/10,000 NaN_3 in the late high blastula stages proceeded only to middle gastrulae.

Discussion and Conclusions

Within one hour after fertilization the oxygen consumption of the *Fundulus* egg persists at a rate which tends to rise in a continuous, but not constant, fashion throughout early development. In agreement with Amberson and Armstrong (1933), no evidence has been found for a transient rise in respiratory intensity to a temporary maximum at about

one and one-half hours after fertilization, as might be expected from the work of Boyd (1928). Moreover, the continuous rise in the rate of oxygen consumption per egg throughout early development is in disagreement with the conclusion of Hyman (1921) that at mid-gastrulation the rate of oxygen consumption per unit of protoplasm is at its highest level for all development. Further increases in rate per egg do occur after mid-gastrulation and these increases are part of a trend in a positive direction which is in evidence before, during, and after the mid-gastrulation period.

It is clear that the rate of increase in respiratory intensity per egg is not constant but varies at different times during development. The first phase of the increase may be correlated with the rapid division of the original one-celled protoplasmic cap into the advanced blastula of many cells. After commencement of gastrulation subsequent increases may primarily result from the addition of new cells to the active embryonic mass by assimilation of yolk material.

In a few cases cell counts have been made of *Fundulus* embryos during early developmental stages (Richards and Porter, 1935; Jones, 1939). In an embryo corresponding to a late high blastula (Stage 9 or about 11 hours after fertilization) there were found about 1,300 cells. Another embryo, an expanding blastula with evidence of a germ ring (Stage 11 or about 18 to 20 hours after fertilization), had about 27,000 cells. For the next 10 to 15 hours the increase in cell number is surprisingly small. At the time of blastopore-closure (Stage 15; 30–35 hours) the total count is at most 35,000 cells. Subsequently the cell number rose relatively rapidly and at the end of the second and third day was about 60,000 and 150,000 respectively.

These data indicate that prior to gastrulation there is a rapid increase in cell number. During gastrulation the total number of cells increases very little. After gastrulation the increase is again quite rapid. There seems to be an obvious correlation between the increase in cell number and the rapid rise in rate of oxygen consumption prior to gastrulation, the falling off during gastrulation, and the rapid rise again after gastrulation.

The results of Trifonova (1937) suggest fundamental differences in the development of perch and of *Fundulus* eggs as far as respiratory rates are concerned. Perch eggs appear to undergo sharp increases in oxygen consumption followed by equally marked decreases at the commencement of gastrulation. Similar fluctuations occur during the time of first appearance of the embryo. Trifonova has concluded that the observed high respiratory rates are directly correlated with fundamental physiological changes occurring during periods of differentiation. On

the basis of the present experiments this is not true for *Fundulus*. It is interesting for comparison to note that the results of Brachet (1934), Atlas (1938), and Steffanelli (1938) with amphibian eggs are in agreement with *Fundulus* in showing a continuous rise in respiratory rate per egg without sudden fluctuations during early development.

The demonstration of a constant cyanide-insensitive respiration in the *Fundulus* egg is of some interest. The relative constancy of its absolute value during the first four days of development would tend to indicate that the respiratory increases in early development are primarily the result of increases in the cyanide-sensitive enzyme system. Similar changes in the absolute value of cyanide-sensitive respiration have been found in various developing eggs. The increased respiration caused by fertilization in the sea-urchin eggs is known to be cyanide-sensitive (Runnström, 1930, 1935; Örström, 1932; Korr, 1937). Likewise the respiration of grasshopper eggs in the diapause state is cyanide-insensitive while the difference in respiratory rate between diapause and developing eggs is the result of the relative absence or presence of a cyanide-sensitive oxidation system (Bodine and Boell, 1934).

Since the work of Fisher and others mentioned above has demonstrated the presence of cytochrome-oxidase in the pace-maker mechanism of the embryonic *Fundulus* heart, it is not too great an assumption for the present to state that the probable effect of cyanide on the respiration of *Fundulus* eggs is mediated through the reversible combination of HCN with cytochrome-oxidase. Such combination, by inhibiting the reduction of ferri-cytochrome-oxidase, reduces the oxidations within the cells of the egg to the level of the low cyanide-resistant respiration. Furthermore, the development of *Fundulus* eggs treated with cyanide as described in the results of this paper is similar to the development of eggs subjected to anaerobic conditions as demonstrated in the experiments of Loeb. This is also true in solutions of NaN_3 which inhibit cytochrome-oxidase activity in a manner similar to NaCN. From the close agreement between the work of Loeb and the work presented in this paper, it seems that the effect of NaCN and NaN_3 on development of *Fundulus* is produced by curtailment of cellular oxidations as is true for the effects of anaerobiosis.

Although no attempt was made to ascertain the effect of NaCN or NaN_3 on the respiration of the pelagic eggs, it seems valid to assume that the poisons act by inhibiting cellular oxidations in these eggs. This assumption is again substantiated by the similarity of the effect of NaCN and NaN_3 in suitable concentrations and the effect of oxygen lack as shown in Loeb's experiments with cunner eggs.

The question now arises as to the significance of the cyanide-resistant respiration in development. It is not likely that the development of the *Fundulus* egg in the cyanide or azide solutions can be attributed to the presence of a cyanide- or azide-stable respiration for in Loeb's experiments no oxygen was available to the eggs. In spite of this fact the eggs were able to develop under conditions where a cyanide-stable respiration was of no significance. Furthermore, available comparisons of development of *Fundulus* under the different conditions of the two types of experiment reveal that eggs placed in M/1,000 NaCN shortly after fertilization developed no further than did similar eggs under anaerobic conditions. Obviously there is no evidence to show that the cyanide-stable respiration, which is a considerable proportion of the total respiration in the first day of development (20 to 30 per cent), plays any rôle in the developmental processes of the *Fundulus* egg. No positive, quantitative statements can be made with regard to the possible rôle of the cyanide-stable respiration until parallel experiments using oxygen-lack and cyanide are compared simultaneously on similar batches of eggs. For the present, however, it would appear that in the *Fundulus* egg the particular aerobic processes upon which development ultimately must depend involve the cyanide-sensitive respiratory system. It would not seem apparent that any non-specific respiratory mechanism can provide the necessary energy source for the development of the embryo.

An interesting problem which grows out of these investigations is the mechanism of anaerobic development. Brachet (1934) has suggested, with some supporting evidence, that an oxidative reserve is present in the developing amphibian eggs. This reserve would be some sort of hydrogen-acceptor which could carry out oxidations within the developing egg during anaerobiosis. Whatever the nature of the anaerobic source of energy necessary for development in the *Fundulus* egg, it is clear that under conditions of different rates of oxidation, presumed to be the result of using different concentrations of inhibitors, the energy source is depleted at correspondingly different times. Thus in more dilute poison, eggs presumably respire at greater rates and as a result reach later stages before complete inhibition of development results or else continue development with decreased velocities.

From such results it is conceivable that development depends directly on anaerobic reactions involving the presence of some substance whose synthesis is primarily aerobic. Furthermore, this synthesis seems specifically performed by the cyanide-sensitive respiratory system as indicated above. Anaerobic development may then be assigned to the presence of an excess amount of this substance. Moreover, lowered developmental rates under conditions of reduced respiration within the

egg may be thought of as steady states arising from the continuous depletion of the necessary substance and its continuous but retarded rate of synthesis. Under such circumstances the limiting factor in the developmental rate will be the steady state concentration of the substance.

From this viewpoint there is a possible similarity in the action of the inhibitors of cellular respiration on pelagic eggs and on the *Fundulus* egg. It is apparent that the pelagic eggs cannot withstand large reductions in oxidative intensity without undergoing almost immediate inhibition of development. However, the results have demonstrated that in dilute concentrations of poison, where presumably reductions in respiratory rate are not as severe, slow rates of development are possible before complete inhibition occurs. This might mean that in the pelagic eggs as in the *Fundulus* egg there is also some anaerobic source of energy whose utilization becomes manifest during periods of relative anaerobiosis. Or alternately, in contrast to the *Fundulus* egg, the early development of the pelagic eggs may be more directly dependent on completely aerobic mechanisms.

Summary

1. The respiratory rate of the *Fundulus* egg increases during the first two and one-half days of development. There is no temporary maximum rate with subsequent decline shortly after fertilization, nor is there evidence for any striking fluctuations in rate during the period studied.

2. The increases in cellular oxidation in the early development of *Fundulus* may be attributed to increases in cell number and in the amount of material incorporated in the active embryonic mass. These increases in rates of oxidation are not to be correlated with any particular embryonic stage.

3. The respiration of the developing *Fundulus* egg is cyanide-sensitive. The absolute value of the insensitive portion of the respiration remains relatively constant through four days after fertilization. Therefore, the respiratory increases occurring during development are almost entirely in the cyanide-sensitive respiratory system.

4. The earlier observations of Loeb on the relative sensitivity of the *Fundulus* and cunner eggs to anaerobic conditions are confirmed by the use of the respiratory poisons, NaCN and NaN_3. In their sensitivity to these poisons scup and mackerel eggs resemble those of the cunner.

5. *Fundulus* eggs before the end of gastrulation are capable of extensive development in high concentrations of NaCN and NaN_3 which completely and almost immediately inhibit the pelagic eggs. In lower concentrations the pelagic eggs can develop at decreased rates.

6. No differences in sensitivity to cyanide or azide have been found among the eggs at various embryonic stages studied in this paper.

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